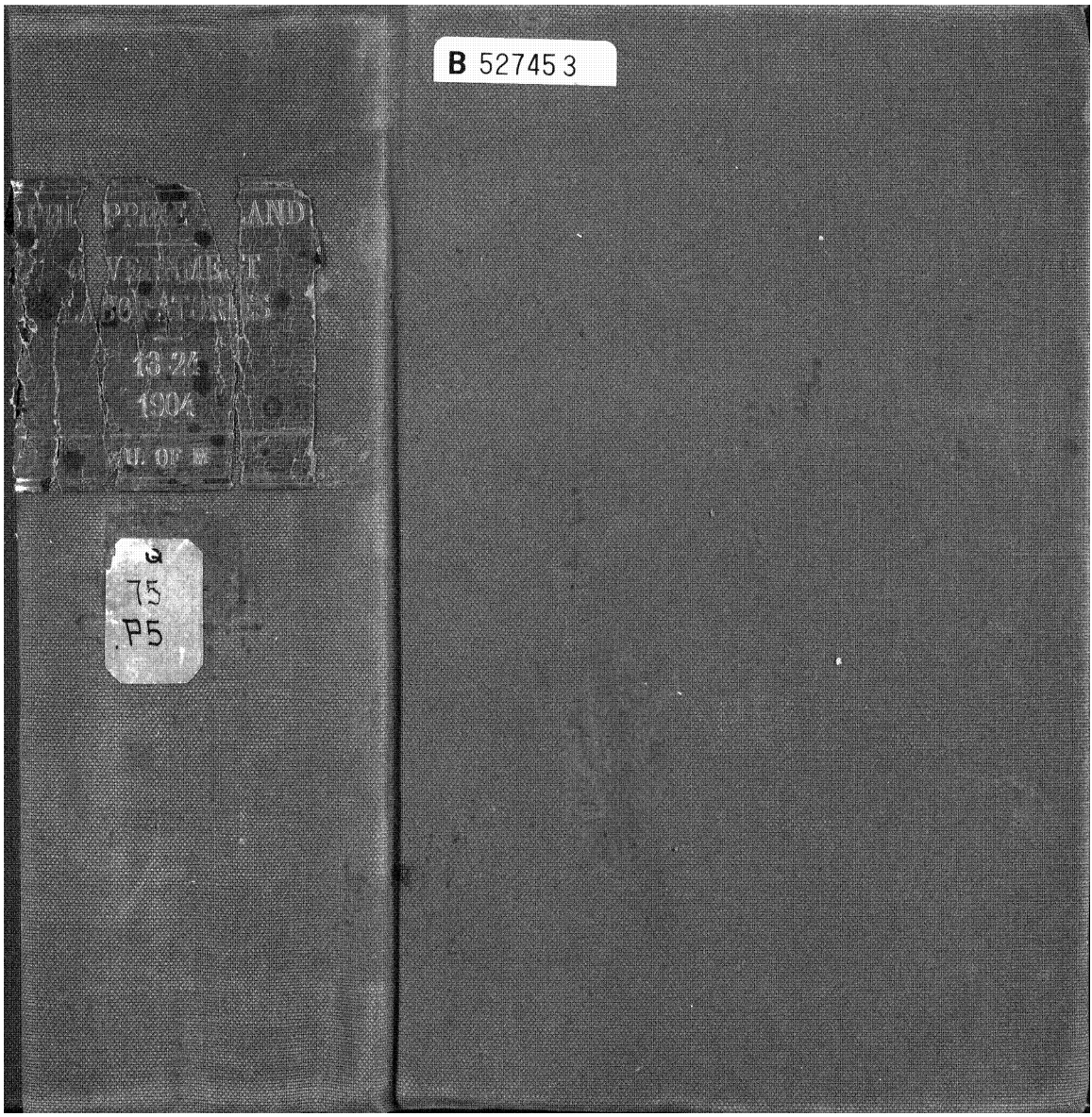

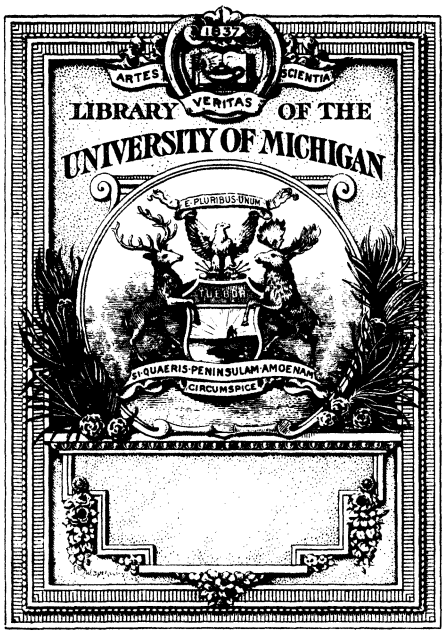

No. 21.—OCTOBER, 1904

DEPARTMENT OF THE INTERIOR
BUREAU OF GOVERNMENT LABORATORIES
BIOLOGICAL LABORATORY

# SOME QUESTIONS RELATING TO VIRULENCE OF MICRO-ORGANISMS, WITH PARTICULAR REFERENCE TO THEIR IMMUNIZING POWERS

BY

RICHARD P. STRONG, M. D.

MANILA
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# LETTER OF TRANSMITTAL.

DEPARTMENT OF THE INTERIOR,
BUREAU OF GOVERNMENT LABORATORIES,
OFFICE OF THE SUPERINTENDENT OF LABORATORIES,
*Manila, July 15, 1904.*

SIR: I have the honor to transmit herewith and recommend for publication a paper, entitled "Some Questions Relating to the Virulence of Micro-Organisms, with Particular Reference to Their Immunizing Powers," by Richard P. Strong, M. D., Director of the Biological Laboratory.

I am, very respectfully,

PAUL C. FREER,
*Superintendent Government Laboratories.*

Hon. DEAN C. WORCESTER,
*Secretary of the Interior, Manila.*

# SOME QUESTIONS RELATING TO THE VIRULENCE OF MICRO-ORGANISMS, WITH PARTICULAR REFERENCE TO THEIR IMMUNIZING POWERS.

By RICHARD P. STRONG, M. D., *Director Biological Laboratory.*

The experimental work which forms the basis of this article was for the greater part performed during the spring of 1903, in the Institut für Infektionskrankheiten, Berlin, Prof. R. Koch, director, department of Prof. A. Wassermann. I wish here publicly to express my very grateful thanks to Professor Wassermann, under whose direction these studies were first undertaken, for many suggestions and courtesies. I also wish to express my gratitude to my colleague Dr. Paul C. Freer for having read the manuscript.

During an experimental study of protective inoculation against Asiatic cholera it became desirable to conduct experiments with two strains of cholera spirilla of different degrees of virulence. Throughout the course of these studies, which extended over a period of a number of months, there often arose in my mind the question of the essential differences existing between these two stems, particularly in relation to the subject of their virulence and to the immunity to which they gave rise in inoculated animals. The investigation of these questions forms the basis of this paper.

For the description of the two strains—their source, identification, etc.—the details of the technique employed in the experiments, and other particulars, the reader is referred to a previous article (1).

It will be sufficient to state here that some time was spent in accurately standardizing these cultures, and the minimal lethal dose for guinea pigs of 250 grams' weight was carefully determined.[1] After numerous passages of *"virulent"* through animals,

[1]For the sake of brevity these strains will, in this article, be designated as *"virulent"* and *"avirulent."*

the lethal dose of 0.1 of a standard (2 milligrams) *oese* [1] of a twenty-hour agar culture was reached. Such a dose of *"virulent,"* when suspended in 1 cubic centimeter of an 0.85 per cent sodium chloride solution and injected intraperitoneally into a guinea pig of 250 grams' weight, regularly caused death within twenty-four hours; with *"avirulent,"* on the other hand, 1½ standard *oesen* of a twenty-hour agar culture, when injected intraperitoneally, were required to produce death within the same time in such an animal. The former stem, therefore, may be said to possess fifteen times the virulence of the latter.

Throughout the course of the work this relation of virulence was carefully maintained. As the virulence of cholera spirilla which have been grown upon artificial media quickly changes, it was necessary in the case of the virulent organism to make daily animal inoculations and always to use the same generation of the strain. With the avirulent culture considerable care was also required to keep it at the minimal lethal dose of 1½ *oesen.*

## TECHNIQUE.

The technique of the agglutinative and bactericidal reactions employed throughout the work was as follows:

The reactions for agglutination were performed in the test tube. One *oese* of the living organism was thoroughly suspended in 1 cubic centimeter of an 0.85° per cent solution of sodium chloride. The amount of serum to be tested suspended in 1 cubic centimeter of a similar saline solution was then added, the tube thoroughly shaken, and the mixture placed for two hours at 37° C. In a complete agglutination it is understood that the liquid overlying the precipitated bacteria appears entirely clear. By a weak reaction we understand one in which there is a distinct agglutination with precipitation, visible to the naked eye, of numbers of the organisms, but in which the supernatant fluid remains more or less cloudy.

The bactericidal reactions were performed in the abdominal cavity of guinea pigs according to the well-known method of R. Pfeiffer, a hypodermic syringe with a blunt-pointed needle being employed for the injections, and care being taken to avoid any injury to the intestine during the inoculation. The dilutions of the serum were made in normal saline solution. One cubic centimeter of the diluted serum was then added to 1 cubic centimeter of bouillon containing 2 *oesen* of *"virulent"* in suspension, after which 1 cubic centimeter of the resulting mixture was injected into the peritoneal cavity of a guinea pig of 250 grams' weight (or a little less), the animal thus receiving ten times the fatal dose of

[1] This standard *oese* was employed throughout the work.

living organisms. A fresh guinea pig was used for each reaction. The experiment was controlled by microscopic examination of a drop of serum from the abdominal cavity, made immediately and again twenty minutes after the inoculation, and obtained by means of a capillary tube, and by the inoculation of control animals with ten times the fatal dose of "*virulent*" without serum. The result to the animal after twenty-four hours, whether living or dead, was regarded as the final test, though the condition of the organisms in the abdominal cavity after twenty minutes was always carefully noted.

With these explanations we may now turn our attention to the study, between the two stems, of certain of the essential differences in relation to virulence.

By the virulence of a micro-organism we have come to understand its pathogenic capabilities—that is, the extent to which it may harm a susceptible host. The virulence of a bacterium, therefore, represents the sum of its specific injurious influences upon such a being. Some authors differentiate between virulence and toxicity, (2) including in virulence only the infectious capability of the organism—that is, the power to grow and to multiply in the animal body—and in toxicity the ability to produce a specific poison, and the amount of such poison. However sharply such a distinction may be observed in diphtheria and tetanus, in which diseases the infectious process (that is, the propagation of the living bacteria) and the intoxication process (that one due to the action of the soluble nonviable substances set free from the organisms) are quite distinct,[1] and while it is well known that certain strains of diphtheria bacilli may at the same time possess strong infective and but little toxic power (and vice versa), in Asiatic cholera the two processes of infection and intoxication are apparently so closely intermingled that, although theoretically a sharp distinction is possible, practically, in the study of the infection of the animal organism, it is only with difficulty that the two are actually differentiated. Hence, in considering the virulence of a strain of living cholera spirilla, both its infectious and its toxic power may, to a certain extent, be regarded conjointly.

Until recently our ideas as to what specific properties the virulence of an organism depends upon have been very vague and indefinite.

[1] With the exception, perhaps, in diphtheria of the intracellular toxine of Welch and Flexner concerned in the production of the false membrane.

Kruse, (3) in his earlier ideas regarding the theory of infection and immunity, believed that a certain analogy existed between the virulence of micro-organisms and their ability to produce enzymes. He conceived the hypothesis that pathogenic bacteria possess a certain dissolving power (*"lytische Kräfte"*), through which they are able to bind and to paralyze the opposing bodies (alexines) of the living organism. A loss of virulence was therefore supposed to go hand in hand with a loss of these dissolving substances (or *Angriffsstoffe*).

Smirnow (4) pointed out that cultures which show a loss of potency in their vital manifestations (especially in their virulence) also present simultaneously a diminished energy of growth. Thus, he maintained that the diameters of colonies of virulent anthrax bacilli are from two to four times as great as those of avirulent strains of the same organism. However, he shows that the diminished virulence of a bacterium depends not alone upon the loss of some one specific property but upon a real degeneration of the organism, which manifests itself by a diminished energy of growth and a greater susceptibility to damaging influences.

Behring, (5) in working with different strains of anthrax bacilli, in general confirmed Smirnow's observations. He further pointed out that in cultures of this organism the production of acid and the power to reduce litmus increase with the virulence of the strain.

Gotschlich and Weigang (6) maintain that the virulence of a cholera culture depends exclusively upon the number of living individual spirilla which it contains. According to their idea, the virulence of an organism would depend upon its power to multiply within a given time, that is, the more virulent the organism the shorter the interval between successive generations.

Beyer (7) found that if a small piece of silver foil was placed upon the surface of an inoculated agar plate the more virulent the organism the narrower was the clear zone between the growth and the silver foil. In case the organism was very virulent, the growth was said to extend so as to touch the margin of the silver.

Marx and Woithe (8) maintained that the virulence of a micro-organism in a human or an animal infection can be judged by the number of bacteria which contain *Babes-Ernst's* bodies. The more

numerous such organisms are in a disease or in a culture, the greater is its virulence. However, Ascoli (9), Krompecher (10), and Gauss (11) were not able to confirm these observations. Thus, Gauss, with a culture of *Bacillus pyocyaneus,* which had been passed many times through animals, obtained a virulence forty times as great as that of the original strain. Yet he was unable to find *Babes-Ernst's* bodies in a single organism of this culture, which possessed the highest obtainable virulence.

Pfeiffer, (12) in 1897 found that in immunization against plague the degree of immunity produced depended not only upon the dose of the killed pest culture but also upon the degree of virulence of the killed organism. He showed that an ape, which had been given a single injection of a virulent agar culture, carefully sterilized by heating, was later protected against 1 *oese* of the virulent bacterium. If, however, an avirulent strain of the pest culture was employed for the virus, the animal was not protected against the same amount of the virulent germ. Therefore Pfeiffer concluded that the immunizing effect of pest bacilli is up to a certain degree proportional to the virulence of the culture employed. Kolle and Pfeiffer, (13) in 1895 also demonstrated that a virulent typhoid bacillus required many more times the amount of immune serum to bring abount its bacteriolisis in the abdominal cavity of a guinea pig than did the less virulent strain. In 1896 these authors (14) pointed out that an immune serum agglutinated a less virulent cholera organism in much higher dilutions than a virulent one. Pfeiffer (15) continued his researches in this direction, and further investigated with Friedberger (12) the question of the virulence of the cholera vibrio. They concluded that in the case of the killed cultures of this organism the immunizing effects were also proportional to the virulence of the inoculated strain. From these facts they drew the conclusion that the virulent and the avirulent organisms differ in the number or degree of affinity of their haptophore groups; and demonstrated this conclusion by experiments in which it was shown that in a goat's cholera immune serum a virulent organism bound many more times the number of amboceptors than certain avirulent ones.

These last experiments of R. Pfeiffer and Friedberger seemed of such great importance in connection with Ehrlich's hypothesis

that it was decided to repeat them, and, in addition, to perform them in as accurate a comparative way (with relation to the virulence of the strain) as practicable. This seemed desirable because in Pfeiffer's and Friedberger's work, as far as can be ascertained from their article, no attempt was made previously to determine the exact relationship of virulence of the different stems to one another, it being apparently the authors' idea in these experiments merely to show that the virulent organism always bound more amboceptors than the less virulent ones; at any rate, the exact relationship between the virulence and the power of binding amboceptors was not emphasized.

Moreover, Pfeiffer's and Friedberger's absorption experiments above referred to were performed with the serum of immune goats, while the bactericidal reactions were performed in the abdominal cavity of guinea pigs. Since at this time it was not established whether the receptor structure of the cholera vibrio for both of these animals was identical,[1] and whether the difference in virulence between the two strains was relatively the same for each animal, and since it was even disputed whether their intermediary bodies were identical, it was thought desirable to perform these experiments also with guinea-pig serum. This work was undertaken in the following manner:

## ABSORPTION EXPERIMENTS WITH THE LIVING ORGANISMS AND GUINEA-PIG IMMUNE SERUM.

A large guinea pig was inoculated subcutaneously with 2 cubic centimeters of an aqueous solution containing the free receptors of the virulent cholera spirillum obtained by the autolytic digestion of the organism,[2] and eight days later was reinoculated intraperitoneally with 2 *oesen* of the living virulent strain. After another eight days it was killed by bleeding and the bactericidal value if its serum carefully determined. This was found to be about 0.11 milligram. Separate portions of the serum were then

[1] Since these experiments were performed Pfeiffer and Friedberger (16) have shown that goats' and rabbits' cholera amboceptors unite with the same receptors of the cholera spirillum.

[2] This solution constitutes our cholera prophylactic. For a detailed description of its preparation, etc., the article on protective inoculation against Asiatic cholera (1) above referred to should be consulted.

diluted with normal saline solution in the proportions of 1:20 and 1:100. Four centrifuge tubes (designated for convenience as A, B, C, and D) were then taken. Two of them, A and B, were filled with 5 cubic centimeters of the serum diluted in the proportion of 1:20; the other two, C and D, with 5 cubic centimeters of the serum diluted in the proportion of 1:100. Five *oesen* of the living virulent organism were then suspended in the serum of each of tubes A and C, and 5 *oesen* of the living avirulent organism in each of the two remaining ones, B and D. After a thorough mixing of the contents, the tubes were placed for two hours in the ice box. This time was allowed for the binding reaction between receptors and amboceptors to become complete, and the object of keeping the tubes at a low temperature was to prevent any further multiplication of the spirilla. Upon removal of the tubes from the ice box the agglutination of the organisms was apparently complete in all of them. After thorough centrifuging, the clear fluid above was pipetted off and in each case carefully examined for its bactericidal value in the usual manner. Table IX shows these results *calculated for 1 cubic centimeter of the undiluted serum.*

From a study of the serum diluted to 1:20, we see that the portion treated with the virulent strain *afterwards* showed a bactericidal value of 1:500 or about one-seventeenth of that treated with the avirulent one (1:8,500), and upon subtraction of these values from that of the original serum (1:9,000), we see that the virulent organism has bound about $\frac{8}{9}\frac{5}{0}$ and the avirulent one about $\frac{5}{9}\frac{}{0}$ of the amboceptors present.[1] In the case of the serum diluted to 1:100, the bactericidal value of the portion treated with the virulent strain (1:600) was about $\frac{1}{1}\frac{}{4}$ as great as that treated with the avirulent one (1:8,500), the *ratio of absorption* being as $\frac{8}{9}\frac{4}{0}$ to $\frac{5}{9}\frac{}{0}$. Hence, evidently in the cholera-immune serum the virulent organism had usually bound from sixteen to seventeen times as many bacteriolytic amboceptors as the avirulent one; or the haptophore groups of the former spirillum had shown from sixteen to seventeen times as great a power of absorption as those of the latter.

The results of these experiments, showing the values of the sera expressed in units of immunity for 1 cubic centimeter of the

[1] The value of the serum in Tube B (see Table IX) varied with different animals between 1–8,000 and 1–8,500. If we regard 1–8,000 as its value then the ratio of absortion in Tubes A and B is as $\frac{8}{8}\frac{5}{0}$ to $\frac{1}{8}\frac{0}{0}$ or 8.5:1

diluted serum, and the number of units *absorbed* per cubic centimeter may be seen in the accompanying table:

| Dilution of the serum to which the bacteria were added. | Original value[1] of 1 cubic centimeter of the diluted serum. | Value[1] of 1 cubic centimeter of the diluted serum after the absorption of 1 *oese* per cubic centimeter of— | | Number of units absorbed per cubic centimeter by 1 *oese* of— | |
|---|---|---|---|---|---|
| | | *Virulent.* | *Aviru en .* | *Virulent.* | *Avirulent.* |
| 1–20 | 450 | 25 | 425 | 425 | 25 |
| 1–100 | 90 | 6 | 85 | 84 | 5 |

[1] In units of immunity. By 1 unit of immunity we understand the amount of serum which will protect a guinea pig of 250 grams' weight against ten times the fatal intraperitoneal dose of living cholera spirilla. (Throughout the course of the work only the "*virulent*" organism was employed in testing the bactericidal power of the sera.)

## ABSORPTION EXPERIMENTS WITH THE LIVING ORGANISMS AND RABBIT'S IMMUNE SERUM.

Experiments were then performed in a parallel manner with rabbit immune serum, which was obtained in the following manner: A rabbit was inoculated with 6 cubic centimeters (1 cubic centimeter contains the number of receptors obtained from 8 *oesen*) of the heated virulent cholera prophylactic. Eight days afterwards it was killed by bleeding, and its blood serum upon examination was found to have a bactericidal value of 0.04 milligram. Five *oesen* of the living virulent strain were now carefully suspended in 5 cubic centimeters of this serum in the following dilutions: 1:20, 1:100, and 1:500. Five *oesen* of the avirulent organism were also added to each 5 cubic centimeters of the serum in the same dilutions. The mixtures were placed in the ice box for two hours. Upon removal it was found that in the tube which contained the serum in the dilution of 1:500 and the virulent organism agglutination was not entirely complete, and that although the overlying liquid was nearly clear it was not wholly so, and evidently still contained some organisms. In the other tubes complete agglutination of the bacteria had apparently occurred. The mixtures were then carefully centrifuged and the overlying liquid pipetted off and in each instance examined for its bactericidal value by the usual method. The results are recorded in Table X (as calculated for 1 cubic centimeter of the undiluted serum), and are in general similar to those obtained in the case of the guinea-pig serum. The values of the sera expressed in units of immunity for 1 cubic centimeter

of the diluted mixtures and the number of units absorbed per cubic centimeter by 1 *oese* of the organism are as follows:

| Dilution of the serum to which the bacteria were added. | Original value[1] of 1 cubic centimeter of the diluted serum. | Value[1] of 1 cubic centimeter of the diluted serum after the absorption of 1 *oese* per cubic centimeter of— | | Number of units absorbed per cubic centimeter by 1 *oese* of— | |
|---|---|---|---|---|---|
| | | *Virulent.* | *Avirulent.* | *Virulent.* | *Avirulent.* |
| 1–20 | 1,200 | 50 | 1,120 | 1,150 | 80 |
| 1–100 | 240 | 12 | 225 | 228 | 15 |
| 1–500 | 48 | 10 | 45 | 38 | 3 |

[1] In units of immunity.

From this table (and Table X) we see that in the dilution of 1:20 the virulent organism had bound about fourteen times as many amboceptors as the avirulent one ($\frac{230}{240}$ to $\frac{16}{240}$); and in the dilution of 1:100 about fifteen times as many ($\frac{228}{240}$ to $\frac{15}{240}$). However, it must be mentioned (and as may be seen from Table X) that the value of the serum in the dilution of 1:20 after treatment with the virulent organism, varied with different guinea pigs between 1:900 and 1:1,000, and after treatment with the avirulent between 1:22,000 and 1:22,400.[1] If we regard the lower value in each instance as the correct one, then the ratio of absorption must be considered as $\frac{231}{240}$ to $\frac{20}{240}$, or as 11:1. but if the higher values are regarded as correct the ratio is as $\frac{230}{240}$ to $\frac{16}{240}$, or as 14.1.

In the dilution of 1:500 the serum, after treatment with the virulent organism, showed a value of 1:5,000 (0.2 milligram); while that previously treated with the avirulent one showed a value of 1:22,500 (nearly 0.04 milligram). Therefore the value of the latter serum was less than five times as great as that of the former, the ratio of absorption being $\frac{15}{240}$ to $\frac{190}{240}$, or about 12:1. However, the experiments with the serum dilution of 1:500 might be misleading without the following explanation.

As already pointed out, the agglutination of the virulent organism in the dilution of the serum of 1:500 was not complete, even after the mixture had stood for two hours at the temperature of the ice box, and after prolonged centrifuging the overlying fluid was still not entirely clear. Evidently there still remained in this supernatant fluid above the precipitate a few vibrios in which a suf-

[1] With only one animal was this value as low as 1:20,000.

ficient number of the haptophore or agglutinophore groups of the agglutinable substances necessary to bring about the phenomena of agglutination were not yet bound by their respective groups of the agglutinin. When this serum was injected into guinea pigs it clearly carried with it not only the *free* bacteriolytic amboceptors of the serum but also those bound to the bacteria and remaining in the slightly cloudy fluid. These amboceptors, meeting with a suitable complement in the abdominal cavity of the guinea pig (and combined with an additional number of amboceptors if necessary), obviously destroyed the bacteria to which they were already united by their haptophore groups before inoculation; and, in this manner (the amboceptors), being set free, were capable of again unfolding their bacteriolytic action against one *oese* of the fresh living organisms introduced for the regular bacteriolytic test. Hence, owing to the combined action, within the abdominal cavity of the guinea pig, of the *free* amboceptors in the serum introduced and of those carried in with it *bound to the bacteria,* the value of the serum appeared higher than it would have done if the agglutination of the organisms by it had been complete and they had been in this manner originally separated from the mixture.

In support of this argument it may be seen from the experiments shown in Table X that, while in the dilution of the serum of 1 : 500 after treatment with the virulent strain (where agglutination was not complete), we find a bactericidal value of 0.2 milligram (1 : 5,000), in the dilution of 1 : 100, after treatment with the virulent organism (where agglutination was originally complete) we find a value of only 0.8 milligram (1 : 1,200).

The actual bactericidal value of this serum (dilution of 1 : 500) in which the agglutination of the virulent organism was not complete could not be accurately determined after the removal of the incompletely agglutinated bacteria by filtration, since in this process, while the combined amboceptors were separated, a portion of those unbound also remained behind on the filter.

In this same dilution of the serum (1 : 500) with the avirulent organism, the agglutination of the spirilla being complete on account of the fewer agglutinable receptors necessary to be occupied in order to bring about this phenomenon, no bacteria, binding amboceptors, were carried into the abdominal cavity of the guinea pig, and the value of the serum was practically the same (viz, 1 : 22,500) as in the lower dilutions of 1 : 20 and 1 : 100.

That the explanation thus given is applicable to these results is confirmed by the very important research of R. Pfeiffer and Friedberger (16), published, however, several months after the experiments mentioned above were completed.[1] These authors show conclusively that the cholera amboceptors bound to cholera spirilla were not destroyed, either in the event of the death of the organisms (bacteriolysis) or in that of their subsequent life, but that they were in both instances eventually set free from the bacteria and again became capable of exercising their bacteriolytic power. A catalytic action is therefore suggested.

On comparing the results of Tables IX and X, emphasis is again laid on the fact that in the cholera-immune serum of both rabbits and guinea pigs the virulent organism usually bound from eleven to seventeen times as many bacteriolytic amboceptors as the avirulent one. This difference in the power of binding practically corresponds to the difference in virulence between our two strains, *"virulent"* and *avirulent."* Hence these experiments bear out the hypothesis that the virulence of a living cholera organism is proportional to the number or degree of affinity of its bacteriolytic haptophore groups.

Another point which is demonstrated by this investigation is that the organisms of each strain bind proportionately the same number of amboceptors in the rabbit cholera-immune serum as in that of the guinea pig. This suggests that the cholera amboceptors of rabbits and guinea pigs unite to the same receptors of the cholera vibrio—that is, that the receptors of this organism are identical for both animals. Pfeiffer and Friedberger (16), since these experiments were performed, have shown by an entirely different method of experimentation that the receptors of the cholera spirillum are identical also for goats and rabbits.

The avirulent organism was next passed successively through the abdominal cavities of about twelve guinea pigs and then examined in regard to its virulence. This was found to have considerably increased, since now three-fourths *oese* of the organism produced death in a guinea pig of 250 grams' weight within twenty-four hours. Unfortunately, at this time there was no longer on hand any serum from either of the animals with which the series of experiments given in Tables IX and X were performed, so that a compara-

[1] A brief summary of the results of my experiments was published in *American Medicine*, Vol. VI, August 15, 1903.

tive study could not be made. However, with another rabbit cholera-immune serum, to which were added corresponding amounts, first, of the original avirulent strain (of 1½ *oesen* virulence), and, secondly, of the avirulent strain after about twelve successive passages through guinea pigs (of three-fourths *oese* virulence), it was found that the latter strain was able to bind in the immune serum nearly twice as many amboceptors as the former. In other words, with an increase in the virulence of the organism, an increase in the number or binding power of its haptophore groups had occurred.

It would be interesting to follow this relationship quantatively to its logical conclusion, and to plot the result as a curve. In this way a mathematical basis of the relation between receptors and amboceptors might be obtained and light thrown on the nature of this relation. This work will be shortly undertaken in this laboratory.

## COMPARISON OF THE AGGLUTINATION OF THE VIRULENT AND AVIRULENT STRAIN.

In the work on protective inoculation against cholera, already referred to, it became evident that the avirulent organism was agglutinated by higher dilution of the same sera than the virulent one. This may be seen in Tables I to VIII of this article. However, it is true that sometimes the difference in agglutination varied, owing to the fact that the serum used was not always of the same age; and in case varying amounts of agglutinoid were present, the usual ratio of agglutination between the two stems was lost, because smaller amounts of agglutinoid prevented agglutination from appearing in suspensions of the avirulent than in suspensions of the virulent organism. Theoretically these results could be explained on the assumption that there existed fewer agglutinable haptophore groups in the avirulent than in the virulent strain, and hence a smaller number of uniceptors was necessary to bring about a reaction in the case of the avirulent germ than in that of the virulent one, so that with the former organism agglutination took place in higher dilutions. Likewise when sufficient agglutinoid was present smaller numbers of such modified uniceptors would suffice to bind the receptors of the avirulent strain than would be required by the virulent one. Hence the phenomenon of agglutination would fail with the avirulent organism in lower dilutions than it would in the case of the virulent one. However, even in a fresh cholera-immune serum no such difference as 15 : 1 could ever be demonstrated in the agglutinable power of the two stems—

that is, the avirulent strain was not agglutinated in dilutions fifteen times higher than those necessary in the case of the virulent race. Neither could it be shown in the same sera (used in the experiments of Table X) that the virulent strain bound fifteen times more agglutinin than the avirulent, though it was true that the amount of agglutinin which the former organism appropriated was about four or five times as great. We may then argue that, while the virulent organism contains more agglutinable substance than the avirulent—that is, that its agglutinable haptophore groups are more numerous—its virulence (from an infectious point of view) is not in direct proportion to the amount of such substance, and just as the amount of agglutinin in a serum has not been found to be directly proportional to the degree of (at least the bactericidal) immunity of the host, so it may now be stated that neither is the amount of agglutinable substance directly proportional to the (infectious) virulence of the organism. Obviously we must not lose sight of the fact that, just as in the immunity of the host, two, and perhaps three, factors may enter into consideration, namely, the antitoxine, the bacteriolysine, and the agglutinine, so, in the question of the virulence of a cholera organism, there may also be three substances to be considered, namely, the toxine, the bacterial cell, and the agglutinable substance.

From our study as to what properties the virulence of our two cholera stems depends upon, and from our recorded animal experiments, it seems that it is the power of the cell to bind bacteriolytic amboceptors and to resist destruction (bacteriolysis) as well which is the most important element connected with the death or recovery of the animal (our indicator of the virulence), and that it is the ratio of this power (both to bind and at the same time to prevent bacteriolysis) rather than that of any other which we express, when we state that the virulence of the organisms is as 15 : 1. Therefore, even should the agglutination proceed in a manner parallel to that of the bacteriolysis, it is questionable whether we should expect, *a priori,* that our virulent organism would bind in the immune serum fifteen times more agglutinin than the avirulent; since it has not been demonstrated (even granting for the moment that the amount of agglutinable substance which the organism possesses is a possible preliminary factor entering into the question of the virulence) that the ratio of the amounts of the agglutinable substance contained in the two stems is as 15 : 1. Indeed, in so

far as the amount of this substance could be demonstrated from the action of the strains upon an immune serum, the avirulent organism was never agglutinated in dilutions higher than about five times those which agglutinated the virulent strain. However, Eisenberg and Volk (17) thought that it was doubtful whether the agglutinable substances of an organism could be fully saturated, since they were able to find almost no limit to this power. Moreover, it is well known that the more concentrated the serum in agglutinine, the greater is the quantity of agglutinine bound by the same amount of agglutinable substance. The question of the velocity of the reaction should, therefore, be carefully considered in relation to this phenomenon.

In connection with this subject, the very recent work of Arrhenius (18) is also of interest. This author found that for constant quantities of bacteria, in which equation the amount of free agglutinine = B and the amount of bound agglutinine = C, the following relation exists: $C = \text{Konst.}\ B^{\frac{2}{3}}$ However, if the quantity of bacteria (A) varied, the following equation was found to exist, viz, $\frac{C}{A} = KB^{\frac{2}{3}}$. That is, the absolute quantity of bound agglutinine did not enter into the question, but only its concentration in its solvent—the bacteria. He pointed out that, with a knowledge of the conditions of equilibrium the assumption that the agglutinine exists as a number of substances with binding properties of different degrees of affinity is superfluous, even if the possibility that the agglutinine is a mixture of several active bodies can not be denied.

Arrheninus apparently worked with a single strain of an organism of a certain virulence. It would be very interesting to perform these same experiments with strains of different virulence and with the free agglutinable receptors of such organisms. We might expect, *a priori,* that the same law would apply in such experiments, since by the "quantity" of the bacteria we probably really understand the number of agglutinable haptophore groups, which, in the case of the virulent organism, would be greater than in that of the less virulent strain. Hence $\frac{C}{A}$ would vary with the virulence of the organism.

However, we must return to the question of the virulence of the two strains of cholera spirilla and defer for the present any further discussion of this matter.

## ABSORPTION EXPERIMENTS WITH THE KILLED ORGANISMS AND RABBITS' IMMUNE SERUM.

With the hope of throwing more light on this relation, a study of the effect of the absorption of the amboceptors from the same immune serum by means of the killed organisms was undertaken. The minimal lethal dose for guinea pigs of the spirilla killed with chloroform was first carefully determined for each strain. It was found that about 5 or 6 *oesen* of the killed virulent organism, when introduced into the abdominal cavity, produced death within twenty-four hours in guinea pigs of 250 grams' weight, while about 8 or 9 *oesen* of the killed avirulent strain were necessary to cause the same result. Therefore, with the killed organisms a ratio of virulence of nearly 2:1 existed.[1] These results speak partly in favor of the hypothesis of Gotschlich and Weigang (6), though from them it is evident that if the virulence of a living organism depended only upon its power to multiply more or less rapidly within a given time, the virulence of our two strains of cholera spirilla (provided that exactly the same amounts of each were used) would probably be equal after the death of the organisms. However, this, as we have just seen, is not the case, a difference in virulence of nearly 2:1 existing between the two killed races. On the other hand, in the living state a difference of virulence between the organisms of 15:1 existed. Therefore, these experiments would support the idea that the energy of growth of an organism is a factor of importance, though not the only one, in relation to its toxic virulence.

For the absorption experiments with the killed organisms the same rabbit cholera-immune serum was used as was employed for those recorded in Table X with the living spirilla.

Five *oesen* of each strain, the *virulent* and the *avirulent,* were suspended in 5 cubic centimeters of bouillon in separate test tubes,

[1] It may be justly argued that this is merely a toxic ratio and not one of virulence. However, if we assume that the toxine is intracellular, the binding power of each strain of the killed organisms for bacteriolytic amboceptors, as will be pointed out further on, is apparently of some importance in the liberation of this toxine, and since the bacteriolytic receptors are partially concerned in this liberation, it has been thought advisible to employ the term virulence (in this connection) as representing the injurious influence of the organism upon a susceptible being (the guinea pig). It is admitted that ordinarily the terms virulence and toxicity may with advantage be distinguished from one another.

to each of which were then added fifty drops of chloroform. After the organisms had been killed, the chloroform was evaporated, the sterility of the mixtures demonstrated, and then there were added to each of the two tubes 5 cubic centimeters of the rabbit serum in dilutions of 1:20. Hence the dilutions of the serum in each of the two tubes containing 5 *oesen* of the organisms equalled 1:40. The mixtures were allowed to stand for two hours, and after complete agglutination had occurred in both of them, the clear fluid above was pipetted off and examined in each instance for its bactericidal value. The results of these experiments, calculated for 1 cubic centimeter of the undiluted serum, may be seen in Table XI. We first notice that the receptors of the organism have been seriously injured or diminished through the killing of the spirilla by chloroform; for, whereas the living virulent organism bound about $\frac{2}{2}\frac{3}{4}$ of the bactericidal amboceptors in the same rabbit serum (see Table X), the killed virulent organism was able to bind only about $\frac{1}{2}\frac{9}{4}$ of them.[1] This loss or injury of the receptors apparently progresses to a certain extent with the loss of virulence, the minimal lethal dose of the virulent strain being for the killed organism about 5 or 6 *oesen* and for the living one $\frac{1}{10}$ *oese*. However, while the ratio of virulence between the killed and the living germ may be expressed as about 1:55, the difference in their power to absorb the bacteriolytic amboceptors is not at all in this proportion. Hence it would appear that the relation between the virulence of the killed organism and that of the living one, both belonging to the same strain, is not always, at least, dependent upon the number or binding power of the bacteriolytic haptophore groups possessed by each, or at any rate not dependent alone upon this condition. In other words, it would seem that in the death of the bacteria by the process described a certain change has taken place in the organism, so that while the receptors may be able to bind in vitro a considerable number of amboceptors, the poisoning action of the bacteria in the animal body is not unfolded to the extent which might be expected. Therefore the idea is suggested that in the killing of the organism with chloroform the intracellular toxine has also suffered a change—that is, it now has been placed in such a condition as not

[1] In making this comparison, we must assume that the living bacteria in the experiments of Table X had not multiplied during the time that they were in contact with the serum (two hours) at the temperature of the ice box.

to be so easily set free from the bacteria (possibly through a retarding of the action of the ferments of the organism), or it has actually been altered chemically. In other words, in the case of the killed bacterium entirely another factor besides its power of binding amboceptors and its resistance to destruction (bacteriolysis) would seem to enter principally into the question of virulence (or the fate of the inoculated animal), namely, the condition and the amount of the intracellular toxine set free.

We have seen that with the killed virulent organism amounts as large as 5 or 6 *oesen* are necessary to bring about the death of a guinea pig. On the other hand, it has been demonstrated by the experiments in which the killed organisms were added to the immune serum, *in vitro,* that the killed bacteria still contained a considerable number of haptophore groups, as the proportion of the amboceptors removed from the serum demonstrates. Indeed, they were capable of binding many more amboceptors than would be necessary in order to bring about their complete dissolution in the animal body. Hence upon their introduction into the abdominal cavity of an animal, probably the factor of the greatest importance in relation to its death or recovery would be the condition and the amount of the intracellular toxine which the bacteria contained. The virulence in this instance would chiefly depend upon this value.

Possibly the slight and partial injury by chloroform of the agglutinophore group of the agglutinable substance in the dead bacteria may also be a preliminary factor which exercises a retarding effect upon the virulence, by placing the organism in a less satisfactory condition for the liberation of the toxine.

After a comparison of the values of the sera following treatment, first with the living, and then with the killed *avirulent* organism (see Tables X and XI), we are inclined to the same explanation, though some of the results can not be satisfactorily interpreted. Thus it must be stated here that even when a smaller number of receptors (than was contained in five *oesen* of the avirulent organism) was placed in this immune serum it was found afterwards that generally a loss of amboceptors of from about 0.001 to 0.003 milligram per cubic centimeter had occurred in the undiluted serum—that is, when the number of receptors existing in the avirulent organism became very small, they seemed endowed, upon

being placed in a concentrated serum, with a slightly greater binding power. Thus, in this series of experiments, with the avirulent organism the killed germ absorbed apparently the same number of amboceptors as the living one, though in each case this was actually very small. However the ratio of virulence of the living germ to the killed one was about $5\frac{1}{2} : 1$ ($1\frac{1}{2}$ to 8 or 9 *oesen*).

On comparing the value of the serum added to the killed virulent organism with that of the one added to the killed avirulent organism, we see that the former has about one-fourth the strength of the latter; or, that the virulent organism has bound $\frac{1}{2}\frac{9}{4}$ and the avirulent one $\frac{2}{2}\frac{}{4}$ of the bacteriolytic amboceptors of the serum. Beginning, then, with a ratio of virulence of about 2 : 1, we obtained an absorption ratio of (bacteriolytic) amboceptors of about 9 : 1—that is, the virulent organism bound nine times as many amboceptors as the avirulent one. However these results are not confusing since it has already been pointed out that the ratio of 2 : 1 is mainly a ratio of the toxic haptophore groups, while that of 9 : 1 is a ratio of the bacterial haptophore groups of the two strains. But as has been pointed out above, even when such an absorption of the amboceptors by these respective organisms occurs in the animal body, an absorption which is evidently far greater than that necessary for the complete dissolution of the bacteria and the liberation of the toxine, the death of guinea pigs will not result with smaller doses than 5 or 6 *oesen* of the killed virulent organism, and 8 or 9 *oesen* of the killed avirulent one, for the reason that in smaller amounts of the bacteria (killed after this manner) there is not present a sufficient amount of the unchanged toxine to accomplish this end. This once more forces us to the conclusion that the difference in virulence between the organisms killed with chloroform *in this manner* is not alone dependent upon the number or the binding power of the bacteriolytic haptophore groups, but also upon the number and binding power of the toxic haptophore groups—that is, the amount and the condition of the intracellular toxine present in the organism at the time of its inoculation.

On the other hand, the virulence of the killed organism may depend to a certain extent upon the number or the avidity of the bacteriolytic haptophore groups, since the greater the number present or the greater their binding power, the larger the quantity

of amboceptors excited and then bound (within a given time), and hence, the quicker the complete dissolution of the bacteria and the greater the amount of toxine liberated within a given moment, and therefore the greater the injury to the animal. We have already seen that in the killed virulent strain the bacteriolytic haptophore groups are actually much more numerous or endowed with much greater binding power than in the killed avirulent one, but in this connection it must again be noted that it is not a question of the bacteria being killed by the amboceptors (death has already taken place) but it is their dissolution which we suppose results with the virulent strain within a shorter period of time. Were we considering the living bacteria, the hypothesis would necessarily be somewhat different.

The living virulent organism evidently has greater powers of resistance than the avirulent, and more amboceptors are required for its destruction, but through the possession of an increased binding power in its haptophore groups, and hence its greater avidity for amboceptors, it is more capable both of appropriating and of giving rise to the production of these groups in the animal body than is the avirulent germ. Therefore, upon the entrance of the virulent bacteria into a susceptible individual a number of the organisms become more quickly destroyed by means of this power to absorb whatever amboceptors are present or are produced at the time of their introduction. The intracellular toxine of these organisms is thus liberated. However, since a sufficient number of amboceptors to satisfy the avidity of all the organisms is not at once produced, the remaining living bacteria during this latent period multiply rapidly through the increased energy of growth which the virulent organism possesses. As soon as the animal body has responded to an additional production of amboceptors or a sufficient number are set free from the bacteria which have already been killed, an additional number of the virulent organisms are bound and destroyed, and a fresh intoxication of the host results; Hence, the virulence of the living cholera spirillum depends, probably, both upon its power of resistance to the amboceptors and its power to excite and to absorb these substances as well as upon the amount of intracellular toxine (the number of toxic haptophore groups) it possesses and its energy of growth. In this connection it is well to call attention to the work of Von Dungern (19) who concluded, from a series of inoculations in animals, that the viru-

lence of two strains of cholera spirilla was independent of their toxic properties. However, it hardly seems that one would be justified in drawing such a conclusion from Von Dungern's experiments. It would appear, at least from his results with the intraperitoneal inoculation of guinea pigs with the killed organisms, that the virulent organism was more toxic than the less virulent one, though it is true that there was certainly a great difference in the ratio of virulence when compared with the ratio of toxicity, the latter being nearly identical. These results, however, practically coincide with our own, namely, that with a difference of virulence of 15:1 with the living organisms, we obtained a toxic ratio with the dead strains of less than 2:1.

It now seemed desirable to study the effect upon this immune serum of the free bacterial receptors of the cholera spirillum in solution, obtained by autolytic digestion and prepared both from the virulent and the avirulent strain.

## ABSORPTION EXPERIMENTS WITH THE FREE RECEPTORS OF THE ORGANISMS AND RABBIT'S IMMUNE SERUM.

Accordingly, 5 cubic centimeters of the virulent and 5 cubic centimeters of the avirulent cholera prophylactic were each mixed separately with 5 cubic centimeters in dilutions of 1:20 of the same rabbit immune serum employed in the experiments comprising Tables X and XI. After allowing the mixtures to stand for two hours, only a very faint precipitate had taken place, though the fluid above became slightly cloudy in both of the tubes, and more so in the one treated with the avirulent prophylactic than in the other. Prolonged centrifuging did not clear the overlying liquid; and in this condition it was pipetted off from the two tubes and examined separately for its bactericidal properties. (See Table No. XII.)

The serum after treatment with the virulent prophylactic showed a value (calculated for 1 cubic centimeter of the undiluted serum of about 0.07 milligram (1:14,000), while that treated with the avirulent one showed a value of about 0.04 milligram (1:23,000). The virulent prophylactic had apparently absorbed about $\frac{1}{2}\frac{0}{4}$ of the amboceptors present and the avirulent one somewhat less than $\frac{1}{24}$, a ratio of absorption of about 10:1. However, these results must be regarded with caution, since we were here probably encountering conditions much the same as those seen in the experiments performed

with the living organism in the higher dilutions of the serum; that is, the bacteriolytic amboceptors united to the receptors were present in suspension in the slightly cloudy mixtures. Upon injection of this serum into animals, these amboceptors, after the destruction or solution of the receptors through the aid of the guinea pig's complement (or of more amboceptors), were not destroyed but again set free; and once more uniting with the receptors of the freshly introduced bacteria evidently gave to the newly added serum an apparently higher bactericidal power. Therefore, the value of the serum after treatment with the virulent prophylactic is probably actually somewhat lower than 0.07 milligram (1 : 14,000); and the same may probably be said of the value of 0.04 milligram (1 : 23,000) of the serum after treatment with the avirulent strain; although the precipitation of the receptors was probably more complete in this instance than in the case of the virulent prophylactic. Furthermore, in the higher dilutions a smaller number of the combined amboceptors was obviously carried into the animal body. It was impossible to obtain accurate results upon the filtration of the fluids, through very dense filters, for, while this process removed the combined amboceptors and receptors, it also removed, as was shown by a control experiment made with the immune serum alone, a considerable number of the free amboceptors. The more concentrated the serum the greater the number of amboceptors removed by filtration. On the other hand a coarser filtration with filter paper did not separate the combined receptors in suspension. Therefore, this series of experiments suggests forcibly (although it does not conclusively demonstrate) that the binding power of the two prophylactics (that is, of the free receptors) added to an immune serum is within certain limits proportional, first, to the immunity caused by each after its injection into animals (see Tables III–VII), and second, to the infectious virulence of the respective strain from which it is prepared.

### COMPARISON OF THE IMMUNITY OBTAINED WITH THE FREE RECEPTORS OF THE VIRULENT AND AVIRULENT STRAINS.

We may now compare the immunity produced by the injection into rabbits of the free receptors of the virulent cholera organism with that produced by the injection of those of the avirulent one. These free receptors were obtained, as has already been stated, by the

filtration of the killed cholera organisms, which had been subjected to autolytic digestion for varying periods of time in aqueous solutions. These free receptors in the fluid constitute the cholera prophylactic. The strength of the prophylactic varies in the different series of experiments according to the number of *oesen* of the bacteria digested in each cubic centimeter of the fluid. In prophylactic No I (see Table No. III) 1 cubic centimeter contains the number of receptors obtained by the digestion of one *oese* of the killed organisms. The strength of the prophylactic in the other series of experiments is indicated in Tables IV–VII, where the results in immunity are also shown; the tables are self-explanatory. In the animals of Table VII and in a portion of those of Table VIII the inoculations were made subcutaneously, the rabbits comprising the former receiving injections of the prophylactic in liquid form, and those of the latter the same substance dried in a vacuum and redissolved in saline solution. On comparing the immunity obtained by the injection of the virulent and avirulent receptors from Table III we see that the ratio of bactericidal immunity between the animals inoculated with the virulent and those inoculated with the avirulent prophylactic varies between 3.5 : 1 and 12 : 1. In Table V the sera obtained from the animals inoculated with the virulent prophylactic showed a bactericidal value of about five and one-half to twelve times that obtained from the injection of corresponding amounts of the *"avirulent,"* and in Table VI the animals of the *"virulent"* series developed sera having six to fifteen times the value of those of the *"avirulent"* one. In Table VII, with subcutaneous inoculation (Nos. 399, 400, 423, 184), the proportion is from 1 : 8 to 1 : 11 and in Table VIII, with the dried prophylactic, the relation is from 1 : 1.33 to 1 : 4. The results obtained with the dried prophylactic are certainly not as accurate as those given by the fluid, because of the manipulations to which the powder was subjected; and since they are not in agreement with all of the other numerous experiments, in which the liquid prophylactic was employed, for the purposes of this argument they must be discarded. With this exception the results here reported with the free receptors are in accord with those which have been obtained by other observers who for inoculation have employed strains of the killed organisms of different virulence; namely, that the immunity obtained is within certain limits approximately proportional to the virulence of the inoculated virus.

## COMPARISON OF THE IMMUNITY OBTAINED WITH THE LIVING VIRULENT AND AVIRULENT STRAINS.

We will next turn our attention to the results in immunity obtained by the inoculation of the living *virulent* and *avirulent* cholera spirilla. In these experiments the rabbits were given intravenously one-half *oese* of the living organisms of the respective strains suspended in 1 cubic centimeter of bouillon. Two series of six rabbits each were inoculated, and on the day of the operation in each instance the ratio of virulence between the two strains was verified as 15:1. The results are recorded in detail in Tables I and II, from which we see that by the intravenous injection of the living organisms in quantities of one-half *oese* the ratio representing the bactericidal value of the sera of the animals inoculated with the virulent and the avirulent organisms was never greater than 4.5:1—that is, the virulent organism never furnished a serum more than four and one-half times as potent as the avirulent one. Therefore, it can not be said that the immunity obtained was directly proportional to the virulence of the organisms, since the latter was 15:1 before inoculation. However, with the digested extracts of the organisms of different strains, as we have just seen, and the killed organisms of different degrees of virulence, this may, within certain limits, be said to be the case.

How shall we explain this discrepancy between the virulence of the living organisms and the immunity produced by each? It may be argued that in such a complicated process as immunization the animal cells could not be expected to respond in a proportional manner to such great differences in stimuli, and further that with such doses, as large as one-half *oese* of the organisms, we could not expect the immunity to increase proportionately to the virulence, since the animal cells are capable of responding only to a certain limit in the production of immunity, no matter how great the stimulus, and since the number of amboceptors given off becomes proportionally (to a given stimulus) less and less as one approaches this limit. If one is convinced by such an argument, for which it is true there is considerable supporting evidence, no further explanation is necessary. However, on the other hand, it may be seen from the experiments with the intravenous inoculations of the free bacteriolytic receptors from both strains (see Table VI), where the receptors from 12 *oesen* of the organisms were injected, that

the serum of the rabbits inoculated with the receptors from the virulent strain showed a value from six to fifteen times as great as that of those treated with receptors of the avirulent organism.

In these experiments it is to be noted that the stimulus from the receptors of 12 *oesen* of the virulent strain was equally as great as that from one-half *oese* of the living virulent organisms, as is evidenced by the fact that about the same bactericidal immunity was obtained in the sera of the animals treated with the virulent strain (comprising Tables I and VI); however, it must be observed from the experiments of Table V, in which more receptors were evidently obtained upon a more complete digestion of the organisms, and where the stimulus from the amount of receptors was evidently stronger than that from one-half *oese* of the living virulent organisms, as shown by the value of the sera obtained, the ratio of immunity fell in one instance as low as 5.5 : 1. Yet in the other the ratio stood at 12 : 1. So throughout these experiments (see Tables III to VII), made to determine the comparative value of the sera of more than twenty rabbits, the animals inoculated with the receptors of the virulent strain furnished a serum from three and one-half to fifteen times as strong as that from the animals inoculated with a corresponding amount of those from the avirulent one. However, in only one instance was the low ratio of 3.5 : 1 obtained, the next lowest being 5.5 : 1, and the next 6 : 1 and 8 : 1.

Likewise, turning to results other than our own, we see that Ascher (20) found, upon the intravenous injection of varying amounts of the killed cholera spirillum into rabbits that 1 *oese* gave rise to a bactericidal immunity more than thirty times as great as one produced with one-tenth or two-tenths *oese*. He also observed that, while 2½ *oesen* in two cases gave less than twice as great a bactericidal immunity as 1 *oese*, 10 *oesen* produced a serum of ten times the bactericidal power of the one produced by 1 *oese*. Therefore, while evidently the individuality of the animal is an important factor in the degree of the immunity produced, a fact borne out by the varying results seen in our Tables, and while also it is evident that with very large doses the immunity is not directly proportional to the quantity of the organism inoculated, at least for amounts of one-half *oese* (reasoning from the immunity obtained with the free bacteriolytic receptors and that with the killed organisms) the explanation of the difference of immunity of 4 : 1 as

against that virulence of 15 : 1 is still lacking. At least no explanation given previously would seem to be satisfactory, whether it be solely upon the ground that the cells in the case of the animal inoculated with the virulent strain have already produced the maximum amount of amboceptors of which they are capable, or upon the ground that their limit of production of amboceptors is so nearly reached by a stimulus resulting from a considerably smaller number of receptors thon one-half *oese* of the virulent organism furnishes, that the increased stimulus produced by this amount of the virulent organism gives rise to so small an increase in immunity that its ratio to that produced by one-half *oese* of the avirulent strain is never greater than 4 : 1.

Therefore, while it is admitted that we should not necessarily always expect in our animals an immunity fifteen times as great from the injection of a stimulus fifteen times as powerful, we might anticipate, if we reason from the results obtained by the injection of the killed organisms or their extracts, that a ratio of immunity nearer to 15 : 1 than that of 2.5 : 1 to 4.5 : 1 would be obtained, when amounts not larger than one-half *oese* of the living organisms are injected. Hence it would seem necessary to seek for some other explanation for these results. The idea is suggested that something has happened to the living *avirulent* strain after its injection into the animal which increases its virulence and brings it into greater similarity with the *virulent* one, so that the ratio of 15 : 1 is lessened; the dissimilarity being existent at the moment preceding injection as is evidenced by the fact that the virulent organism will kill in doses one-fifteenth as great as the avirulent. Should the different strains be killed at this moment and injected, or killed and digested and then injected, this change does not take place. The ratio of 15 : 1, as evidenced by the immunity obtained, is within certain proportions retained, hence depriving the avirulent strain of its life would seem to be at least one of the factors which prevented this change in the ratio.

Let us now consider the influence which an animal which has succumbed to an infection with a given bacterium has exerted upon the infecting organism during the period of its parasitic life upon the host, and also the influence which a normal immune serum exerts upon the virulence of a bacterium which has been cultivated in it.

Since the classical observations of Pasteur and his pupils in 1881, (21) we have known that in general attenuated races of bacteria can be reëendowed with lethal properties by successive passages through susceptible animals. Indeed, this is the method usually employed for increasing the virulence of a given bacterium. However there is a limit to this with every organism, and a culture which had attained its highest possible virulence was designated by Pasteur as a "fixed virus" (22).

We also know from many observations that the virulence of an organism may be greatly increased by its repeated inoculation into fresh serum. Thus Roger (23) as early as 1889 reported that streptococci which through cultivation in bouillon had lost most of their energy of growth and virulence would regain these powers when they were repeatedly inoculated in rabbit serum.

Trommsdorf (24) also found that organisms which had been grown in fresh serum showed an increased resistance to bacteriolysis. Dansyz (52) maintained that anthrax bacilli, when inoculated into fresh serum, became surrounded with a sort of mucous covering, which later protected them, to a certain extent, against the action of bactericidal serum.

Metchinhoff and Roux (26) have shown that the virulence of an organism may be greatly increased by growing it in collodion sacs within the abdominal cavity of an animal.

Professor Welch, (27) in his Huxley lecture on recent studies in immunity, in advancing an hypothesis by which might be explained the source, the mode of production, and the nature of certain bacterial toxines, pointed out that "certain substances of the host of cellular origin assimilable by the parasites through the possession of haptophore groups with the proper affinities become anchored to the receptors of the parasitic cell, which, if not too much damaged, is thereby stimulated to the overproduction of like receptors. These excessive receptors of the parasite, if cast into the fluids or cells of the host, are constituted intermediary bodies or amboceptors with special affinities for these cellular constituents or derivatives of the host, which many lead to their production and for which they possess in whole or in part identical receptors. Provided the host is supplied also with its appropriate complements, there result cytotoxines with special affinities for certain definite cells or substances of cellular origin in the host. The contribution of the parasitic cell to these cytotoxines is the

amboceptors; either the parasite or the host may provide the complements."

In considering the condition of the bacterium as well as that of the animal host, according to the hypothesis advanced, the struggle between the bacteria and the body cells in infections may be conceived as an immunizing contest, in which each participant is stimulated by its opponent to the production of cytotoxines hostile to the other and thereby endeavors to make itself immune against its antagonist.

Ainley Walker (28) performed a series of experiments which, so far as they went, supported this hypothesis of Professor Welch. Walker showed that by growing typhoid bacilli in bouillon to which were added increasing amounts of immmune serum (free from the complement), that their virulence and resistance to serum were increased and their agglutinability diminished. In another series of experiments, (29) he found that the progressive passage of typhoid bacilli through fresh bacteriolytic normal rabbit serum mixed with bouillin in the proportion of 1:10 produced a distinct increase in the virulence of the bacilli toward rabbits and guinea pigs, and also increased their resistence to bacteriolytic serum, as shown by the plate culture method.

Welch's hypothesis includes the explanation which Walker gives for the results of his experiments, and also more. According to the idea of the former, certain bacterial antibodies (discharged receptors) are capable, not only of neutralizing the immune bodies of the host, but with aid of the complements also of poisoning the cells of the latter.

Keeping these ideas in view, let us attempt next to trace the biology of an avirulent strain from the moment of its intravenous injection into a nonimmunized rabbit. Upon the arrival of the organisms in the blood stream they quickly disappear and indeed are, we suppose, soon killed, though just how rapidly we do not know. Pfeiffer and Marx (30) assumed that in a short time the cholera spirilla became anchored to the cells of the spleen, the bone marrow, and the lymph glands, since it was in these organs that the specific protective substances were particularly formed. However, in a later series of experiments Pfeiffer (31) was not able to demonstrate conclusively this increased anchoring power for cholera spirilla of the cells of the spleen.

It would seem to be a mistaken idea to suppose that the

immediate destruction of the organisms is in all cases inevitable, since we know, for example, from the injection of avirulent strains in *micrococcus melitensis* into the blood current of monkeys, that the organisms may remain alive for a period of time and then be reobtained in cultures. Yet eventually they disappear and the recovery of the animal results.

We also know that in many infectious diseases (typhoid fever, etc.) the organisms may be isolated by culture from the circulating blood, and yet finally these bacteria become destroyed and the patient survives the malady.

The most important results in this connection which have been obtained with the cholera spirilla are those of Kolle (32). He found that upon injection of one-half *oese* of the living cholera organisms into the carotid of guinea pigs, that blood drawn at intervals of from five minutes to fifteen minutes after the injection contained but few living organisms since when it was inoculated in streaks upon an agar plate only a few and widely scattered colonies developed upon the media. Evidently within this time the majority of the organisms had been destroyed, although some were still alive. In these experiments and at the same period of time, the bacteria were found to be no more numerous in the spleen than in the blood current. From the observation of Pfeiffer's phenomenon in the abdominal cavity of guinea pigs, we also know that in some cases (even when the animal eventually recovers) some of the organisms may remain alive for more than one-half hour after their injection, and it is only later that they become disintegrated.

For the moment then, let us suppose that our organism is of a sufficiently great virulence to be capable of surviving for at least a few generations, and that, while the animal becomes very ill after inoculation, it eventually recovers. These successive generations of the bacterium multiplying in the rabbit serum will, we suppose, rapidly increase in virulence—that is, their haptophore groups will rapidly rise in number, owing to the stimulus received from the occupation of the receptors of the bacteria by the amboceptors of the normal serum. (Compare with Walker's results with typhoid bacilli in fresh normal serum.) That is, reasoning from the well-known biological law of Weigert, an injury to the bacterial cells will be produced and an excessive generation of the receptors result. Hence the final results in immunity in this instance will be much greater than it would if

the successive generations became no richer in receptors than the one existing at the time of inoculation.

On the other hand, let us trace the fate of the virulent organism upon its injection into the circulation. This strain has already reached its maximum virulence and become a "fixed virus"—that is, it is already saturated with haptophore groups. Therefore its few successive generations can become no richer in such groups than the one used for the inoculation, so that the immunity produced can only correspond to, or at least only equal, that which would result from the generation existing at the time of inoculation, multiplied by the number of generations for which the organism survives. Therefore the immunity obtained by the organism of maximum virulence would not be so great, compared with the stimulus, as would that produced in the case of the living avirulent germ. Furthermore, is it not conceivable that if the same stimulus were received by the virulent organism as by the avirulent one, the latter, which is so poor in receptors, would feel the injury more severely than the former, which is so well protected and so rich in these bodies? Hence would not the regeneration, provided an immediate destruction of the organism did not occur, eventually be greater in the case of the avirulent strain? We know that the number of receptors in the virulent organism must be enormous. We can conceive that it may possess many more receptors than would be required to bind all the existing amboceptors in the normal serum. Therefore if there is still an excess of unbound receptors, will this organism be stimulated as strongly to the generation of others as the avirulent one in which no such excess exists?

Such theoretical conceptions are difficult to confirm. In the first place, it does not seem likely that even one division of the cholera spirillum would take place after its inoculation into the blood circulation, since the shortest period within which this organism has been known to divide, at least on artificial media, is about nineteen minutes. We have no observations to show that this phenomenon takes place in a very much shorter time in the animal body. Provided the organisms were just about to divide at the moment following their inoculation, it is questionable whether any of them would be alive to undergo the same process again at the end of nineteen minutes; but, as already stated, we do not know the exact period

at the end of which all of the organisms will have perished. Ordinarily, as we have seen, after an injection into the circulation of rabbits of a small amount of the cholera spirilla, the organisms after a few minutes can be obtained, if at all, only in slight numbers, from small quantities of the blood. This, however, does not necessarily mean that all of the spirilla in the animal body have been destroyed within this period. On the other hand, it would seem probable that the increase in virulence in the avirulent organism would begin at the moment of the inoculation. Theoretically, therefore, in the brief period of time preceding its destruction it would have an opportunity of increasing its haptophore groups and becoming more like the virulent strain. Still, whether this explanation outlined above is the correct one for this phenomenon can not be conclusively demonstrated without further experimental work and we must admit that we are at present unable, in an entirely satisfactory manner, to account for such a small variation in immunity after the employment of two strains of such different degrees of virulence. Perhaps additional light may be thrown upon the solution of this problem by the performance of similar experiments with other micro-organisms than the cholera spirillum.

It seems very evident that Profesor Welch's hypothesis is very applicable to the cholera organism in its relation to infection and immunity, and explains the reason why, as we have seen, it is only with great difficulty we are able to obtain even small amounts of intracellular toxine from our cultures on artificial media. It further explains how, in the animal body (particularly in the mucosa of the human intestine), the organisms, by the binding of suitable amboceptors to their own receptors, are capable of becoming much richer in endo-toxine and indeed of generating a considerable excess of it within a very brief period. Such a process applies also in the immunization of animals with the living organism, though from the observations made on the injection of the cholera spirilla into the blood circulation of animals it would seem that the bacteria do not find in the blood stream, etc., the same favorable stimulus for the production of the toxic receptors as they do in the cells of the mucosa of the human intestine. On the other hand, the proper amboceptors for the production of the bactericidal and agglutinative substances are here encountered. This conception also explains the difficulty which we have experienced (1) in obtaining large amounts of cholera antitoxine, and the relative ease with which

bactericidal substances are produced in the serum of the inoculated animals.

It would seem that the living avirulent strain by some process (not as yet satisfactorily explained in its entirety) is capable of increasing the number of its haptophore groups in the animal body after its injection into the circulation and before its total destruction; so that a relatively higher immunity is obtained by it than is produced when an organism of maximum virulence is employed. In other words, while in the case of the living organisms, a greater immunity is to be obtained from the more virulent strain, such immunity is not necessarily in direct proportion to the virulence of the bacteria used for the inoculation, as is the case, within certain limits, with the killed bacteria or with their free receptors.

## CONCLUSIONS.

The virulent cholera spirillum possesses a greater number of bacteriolytic and agglutinable haptophore groups, or these groups are endowed with a greater binding power for uniceptors and amboceptors than the avirulent.

The number or the avidity of the bacteriolytic receptors possessed by a bacterium is directly proportional to its virulence.

However, the agglutinable receptors do not follow this law—that is, the agglutinable haptophore groups are not necessarily present in the same proportion as the bactericidal ones.

While the energy of growth is probably sometimes an important factor in relation to virulence, other phenomena must also be considered.

The virulent organism is possessed with a greater number of toxic haptophore groups than the avirulent.

The binding power of the free receptors of the organisms for bacteriolytic amboceptors *in vitro* is proportional to the bactericidal immunity produced in animals *by each,* which latter is in turn proportional to the virulence of the organisms from which the receptors were extracted. The binding power *in vitro* of the dead micro-organisms of different virulence for bacteriolytic amboceptors is not in proportion to their toxicity.

The bactericidal immunity obtained by means of the inoculation with the dead organisms of different virulence or their extracts (obtained by autolytic digestion) is proportional to the virulence of the living strains of the bacteria employed.

With the living organisms, while the bactericidal immunity obtained from the inoculation of animals with the virulent organism is greater than that produced with the avirulent, such immunity is not in direct proportion to the virulence of the bacteria introduced.

These conclusions *apply to the two strains of cholera spirilla employed in the foregoing experiments.* Whether they will also hold good with other strains of this spirillum or for micro-organisms in general, must be decided by further experimental work.

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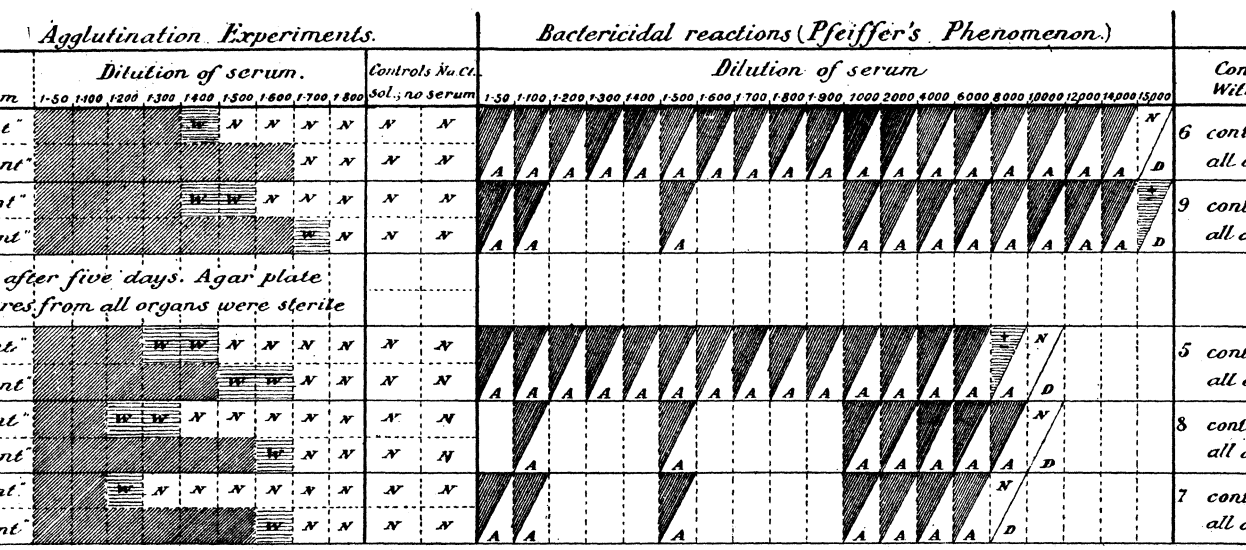
# TABLE No. I

*The average weight of rabbits, 1500 grams.*
*The injections were made into an ear vein with ½ oese of the living organisms suspended in 1 c. c. bouillon. All animals were killed by bleeding one week after inoculation. Agglutination experiments performed with both stems, virulent" and "avirulent"; Bactericidal reactions performed only with the "virulent" stem.*




= *Complete agglutination. Overlying liquid clear.*

*Distinct agglutination with precipitation. Overlying liquid not entirely clear.*

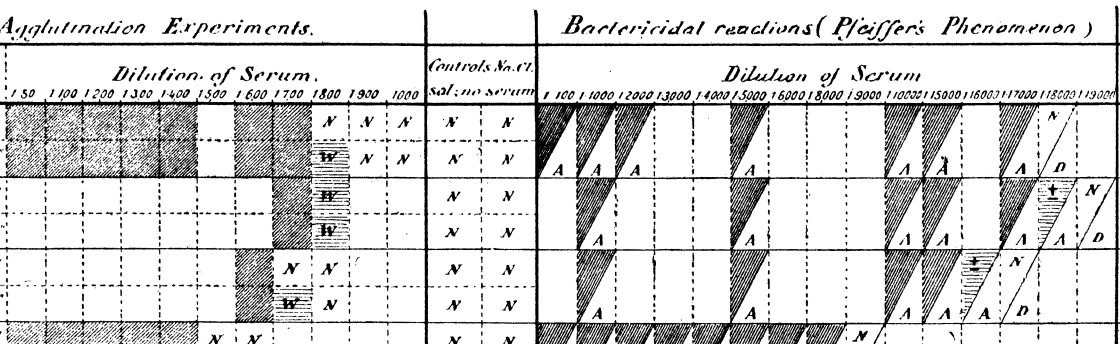
A — *Microscopically, positive bactericidal reaction. Animal alive after 24 hours.*

N/D — *Microscopically, negative bactericidal reaction. Animal dead after 24 hours.*

±/A — *Microscopically, Pfeiffer's Phenomenon doubtful; Animal alive after 24 hours.*

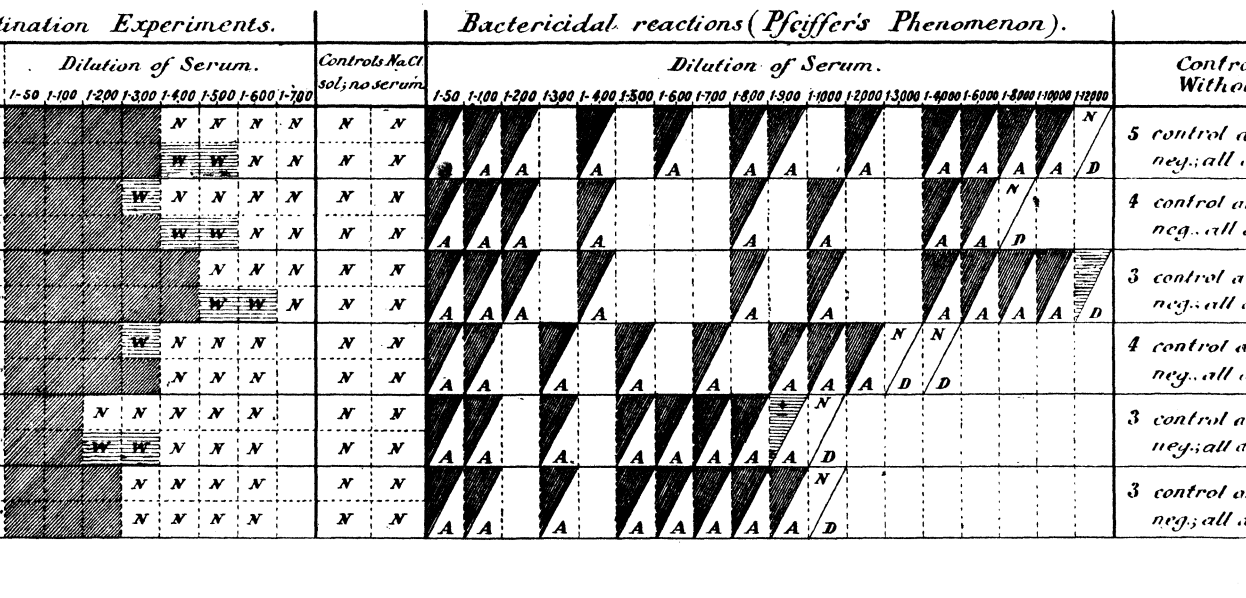
# TABLE No. II.

*Inoculations with living organisms*
*average weight of rabbits 1150 grams.*
*(For explanation see Table No. I.)*



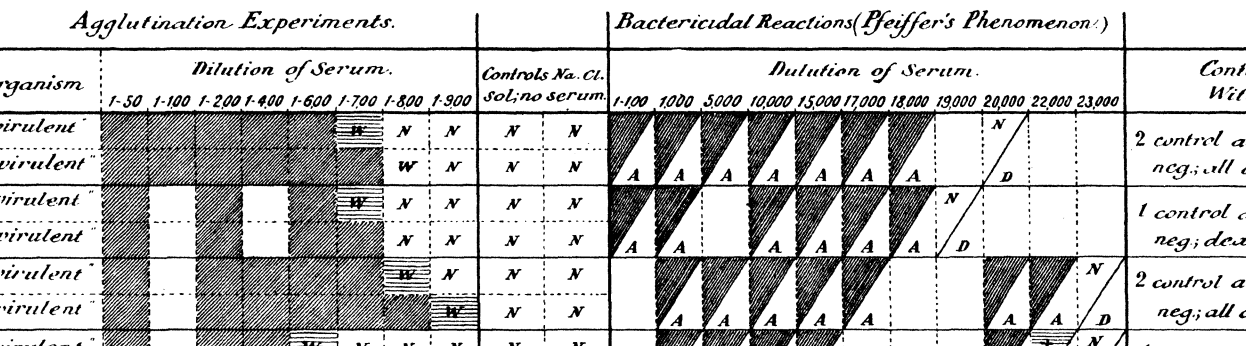
# TABLE No. III.

*Inoculations with "Prophylactic"*
*(For explanation see Table No. I.)*



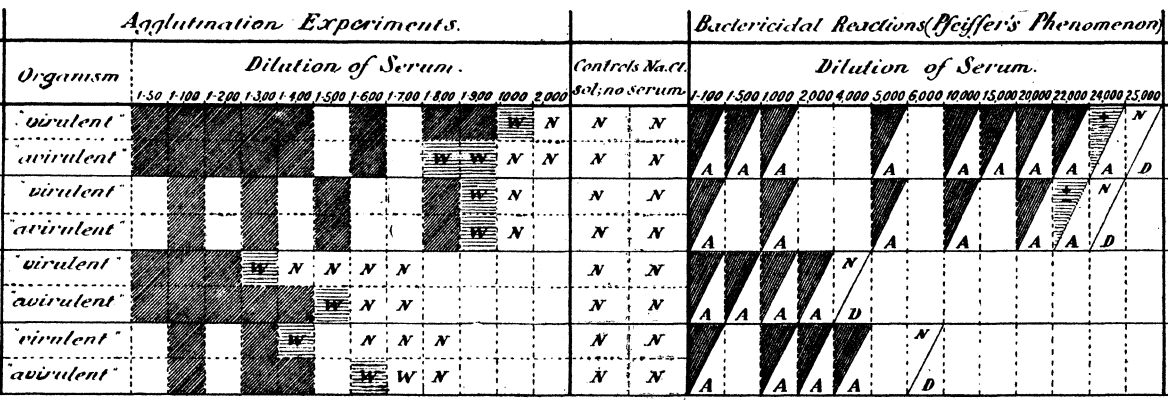
# TABLE No. IV.

*Inoculations with Prophylactic II.*
*(For explanation see Table No. I.)*



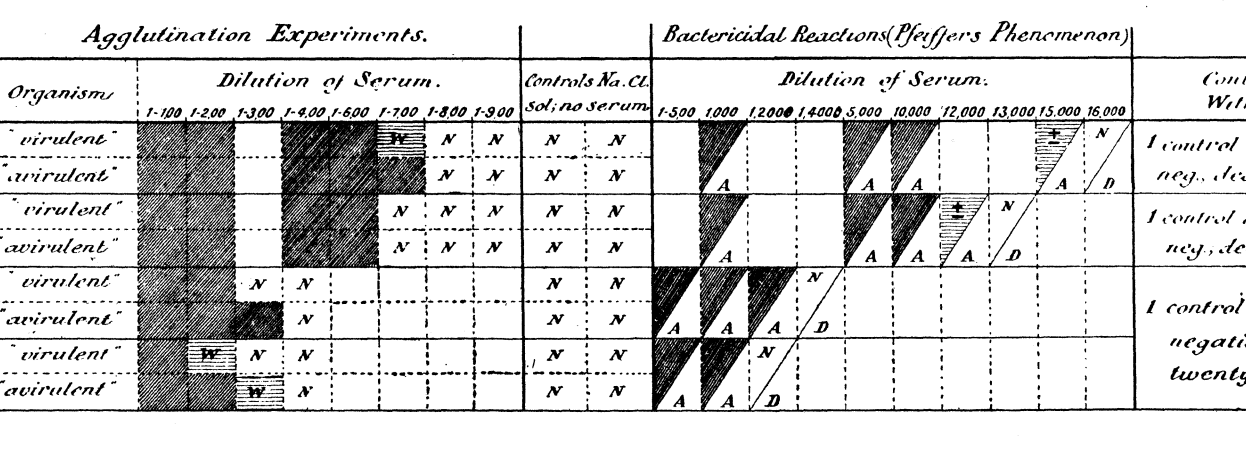
# TABLE No. V.

*Inoculations with Prophylactic III.*
*digested for five days.*
*(For explanation see Table No. I.)*



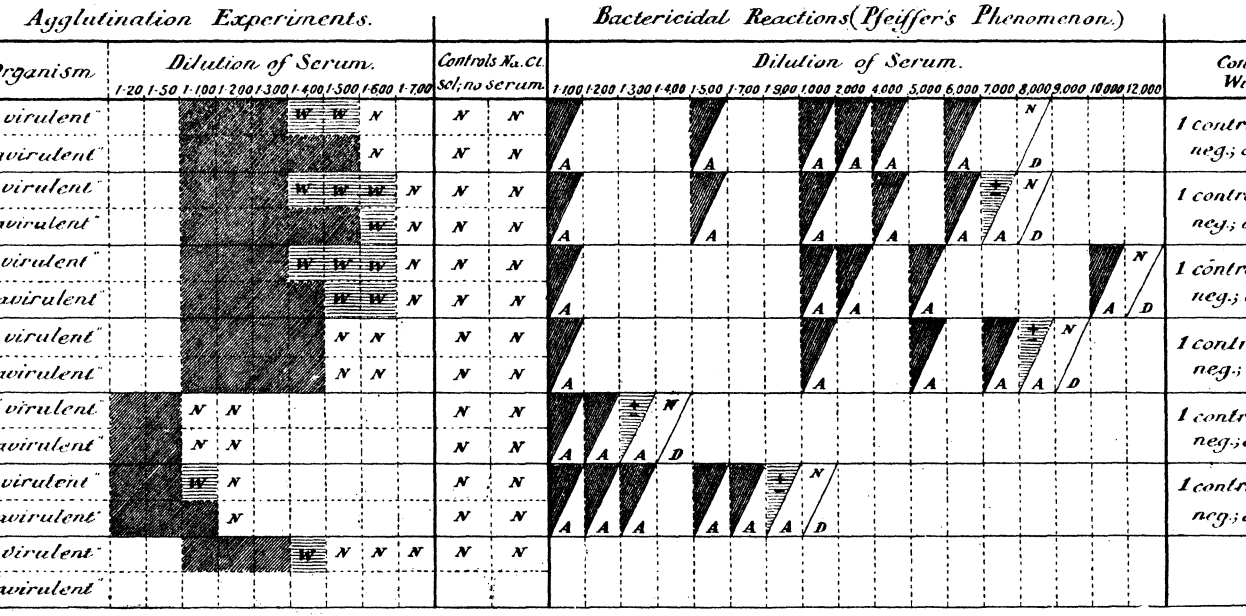
# TABLE No. VI.

*Inoculations with "Prophylactic IV. digested for 2 days.*
*(For explanation see Table No. I.)*



# TABLE No. VII.

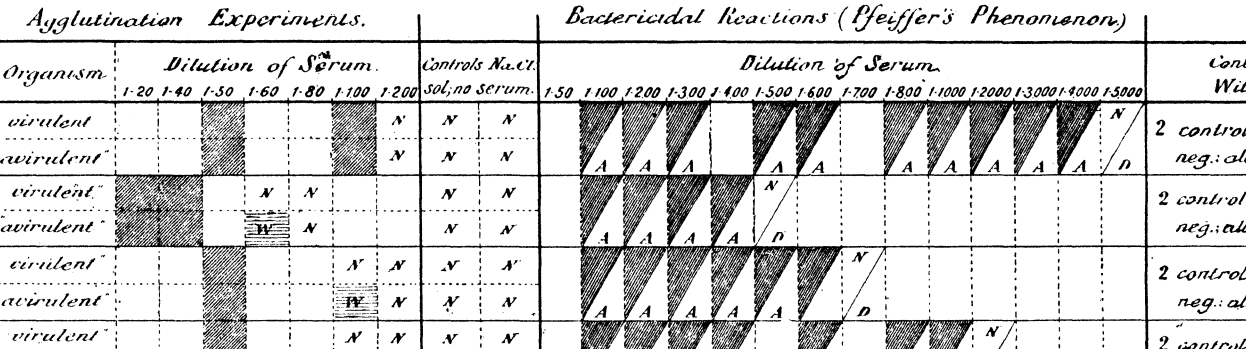
*Subcutaneous Inoculations with Prophylactic*
*(For explanation see Table No. I.)*



# TABLE No. VIII.

*Inoculations with Dried Prophylactic redisolved in Na Cl. sol.*

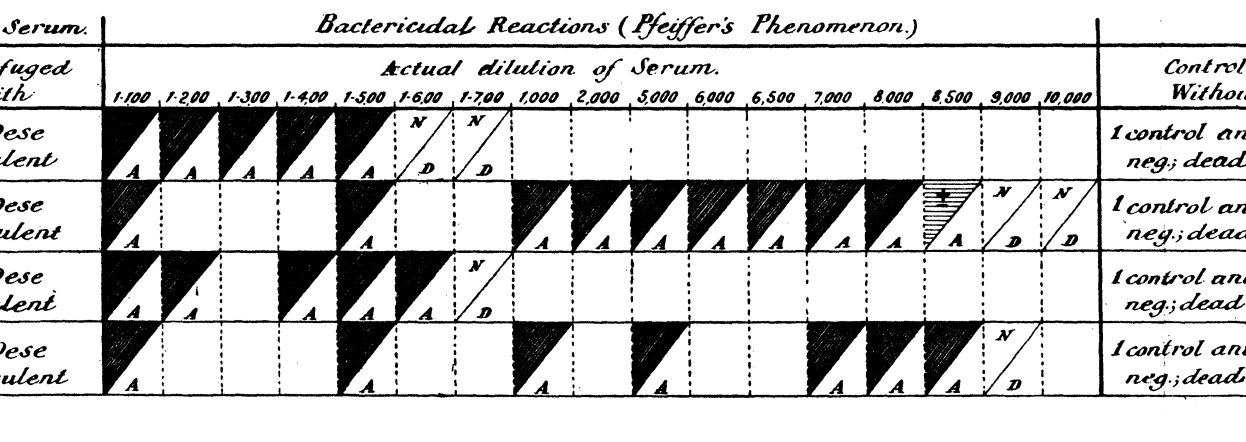
*(For explanation see Table No. I)*



# TABLE No. IX.

*Showing Bactericidal value of Guinea pig serum after treatment for 2 hrs. with the virulent and avirulent strain.*

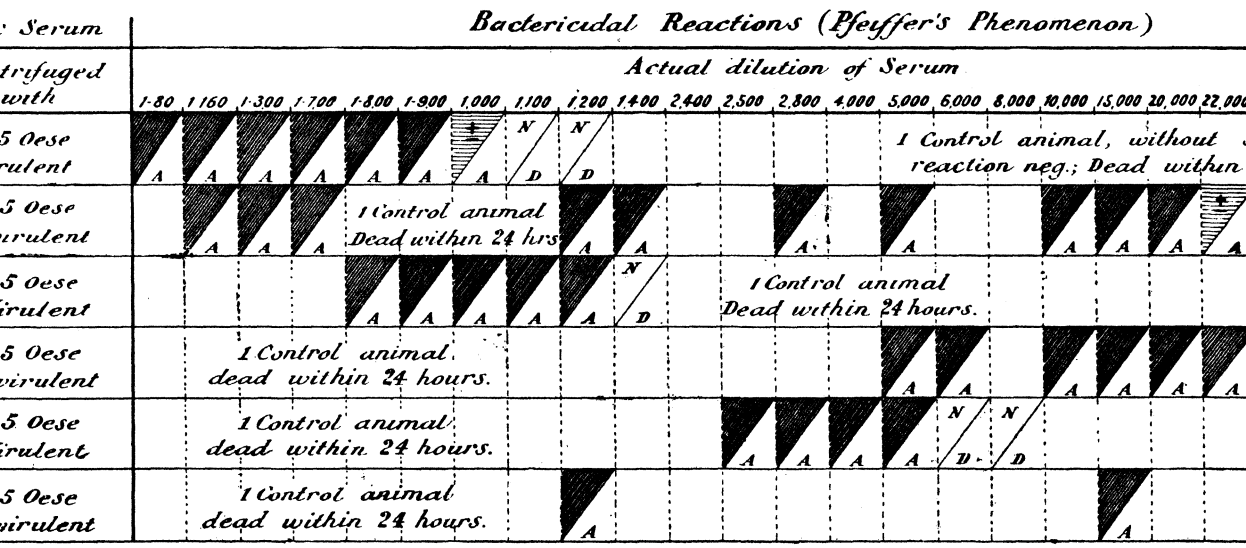
*Original Bactericidal value of Serum...* { *1-9,000 Post. Alive* / *1-10,000 Neg. Dead*



# TABLE No X.

*Showing Bactericidal value of Rabbits serum after treatment for 2 hrs. with the virulent and avirulent strain*

*Original Bactericidal value of serum...* { *1-24,000 Post. Alive* / *1-25,000 Neg. Dead*



# TABLE No. XI.

*Showing Bactericidal value of Rabbit serum after treatment for 2 hrs. with the killed virulent & avirulent organisms.*
*Original Bactericidal value of serum....* { *1-24,000 Post. Alive* / *1-25,000 Neg. Dead.*